

# Motilin Receptors of the Rabbit Colon

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Received 29 June 1990

DEPOORTERE, I., T. L. PEETERS AND G. VANTRAPPEN. *Motilin receptors of the rabbit colon*. PEPTIDES 12(1) 89-94, 1991.—Binding studies with iodinated motilin revealed that in the small intestine motilin receptor density decreased aborally, disappeared in the caecum but returned in the colon and rectum. The highest density was in the distal colon ( $112 \pm 11$  fmol/mg protein). The dissociation constant was the same in all regions (overall mean  $1.10 \pm 0.22$  nM). The ability of erythromycin-A (EM-A) and of two derivatives, EM-A N-oxide and EM-523, to displace motilin showed no difference between the tissues studied. Their order of potency was: motilin > EM-523 > EM-A > EM-A N-oxide. Proximal circular colonic smooth muscle strips showed maximal contractile responses towards motilin, EM-523 and EM-A of, respectively,  $80 \pm 3\%$ ,  $78 \pm 4\%$  and  $84 \pm 2\%$  relative to the maximum obtained with acetylcholine. In proximal longitudinal muscle only a response of  $\pm 20\%$  was obtained. Similar responses were obtained in the distal colon. The order of potency to induce contractions as reflected in the  $pED_{50}$  values was: motilin ( $8.03 \pm 0.1$ ) > EM-523 ( $7.55 \pm 0.03$ ) > EM-A ( $5.84 \pm 0.04$ ) in proximal circular colon. The responses were not blocked by TTX ( $10^{-6}$  M) or atropine ( $10^{-6}$  M), but were reduced by verapamil ( $10^{-6}$  M). The abundance of motilin receptors in colonic smooth muscle, if applicable to other species, opens new perspectives for the therapeutic applications of macrolides with motilin agonist properties.

Motilin      Receptor density      Rabbits      Colon

SINCE the discovery that fluctuations of plasma motilin levels are correlated to the occurrence of the migrating motor complex (9,13), studies on the physiological role of motilin in man and dog have been concentrated on motilin's regulatory role in inducing this pattern.

Little attention has been paid to motilin's effect on the large intestine. Strunz et al. (22) noted that motilin induced contractions in circular muscle strips of the rabbit colon, but not in the taenia coli and in man the situation was reversed. Adachi et al. (1) reported that segments of the rectum of the rabbit oriented in the longitudinal axis responded to motilin, but the response was only 46% of the maximal response to acetylcholine, compared to 102% in the duodenum. In vivo it was found that exogenous motilin stimulated motor activity in the human colon (17) and induced colonic motor complexes (CMC) in the canine colon with a much longer duration than the normal CMC (3). Studies with the motilin agonist erythromycin (14) have also shown that after administration of erythromycin in dog there is not only an effect on the motility pattern of the small intestine but also on the colon. A prolonged contraction occurs in the colon followed by a period of inhibition (16).

In this study we made a detailed exploration of the sensitivity of the rabbit colon towards motilin using two complementary approaches: the in vitro response in an organ bath and binding studies with homogenates. For comparative purposes binding studies with small intestinal tissue were performed as well.

## METHOD

### Reagents

The synthetic nor-leucine<sup>13</sup>-porcine motilin analogue ([Nle<sup>13</sup>-po]motilin) was purchased from Novabiochem (Läufelfingen, Switzerland). Erythromycin lactobionate (EM-A) was a commercial sample made by Abbott Laboratories. EM-523 [de(N-methyl)-N-ethyl-8,9 anhydroerythromycin A 6,9-hemiacetal] developed by Dr. Omura of the Kitasato Institute (Tokyo, Japan) was a gift from Takeda Chemical Industries Ltd. (Osaka, Japan). Erythromycin A N-oxide (EM-A N-oxide) was a gift from Prof. J. Hoogmartens (Lab. of Pharmaceutical Chemistry, University of Leuven, Leuven, Belgium). The structures of these compounds are given in Fig. 1.

### Binding Studies

Binding studies were carried out on homogenates prepared from small intestine, caecum, colon and rectum. The small intestine was divided into six parts of approximately 50 cm length. The colon was divided into a proximal and a distal segment. Smooth muscle tissue was freed from mucosa and serosa, finely minced and homogenized in sucrose buffer with inhibitors (1 mM iodo-acetamide, 1  $\mu$ M pepstatin, 0.1 mM phenylmethylsulfonyl-fluoride, 1 mg/ml trypsin inhibitor, 0.25 mg/ml bacitracin) using a Potter S homogenizer (Braun, Melsungen, FRG) at 1500 rpm for 15 s.

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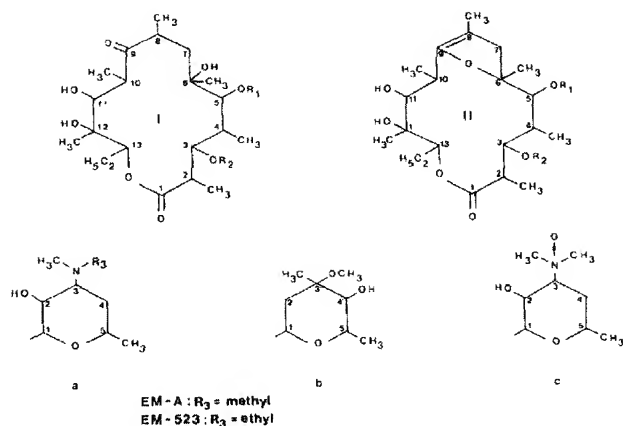


FIG. 1. Structures of erythromycin-A, EM-523 and EM-A N-oxide. EM-A and EM-A N-oxide have ring structure I. EM-A has in  $R_1$  and  $R_2$  the structure (a) and (b), respectively. In EM-A N-oxide the dimethylamino group of (a) is oxidized as shown in (c). EM-523 consists of ring structure II; the same groups are attached at  $R_1$  and  $R_2$  as in EM-A, i.e., (a) and (b) with a modification at  $R_3$ .

Binding of [ $^{125}$ I]-[Nle $^{13}$ -po]motilin was studied in washed  $1000 \times g$  fractions of the tissue homogenates as previously described (4). Briefly, membranes were incubated with [ $^{125}$ I]-[Nle $^{13}$ -po]motilin (specific activity  $\pm 1500$  cpm/fmol, final concentration 50 pM) in 50 mM Tris buffer (pH 8.0, 1.5% BSA, 10 mM  $MgCl_2$ ) for 60'. The reaction was stopped by adding 3.2 ml of cold buffer and membrane-bound motilin was separated by centrifugation at  $1000 \times g$ . All data were corrected for nonspecific binding determined after the addition of an excess of unlabeled motilin. The dissociation constant ( $K_d$ ) and the maximal amount of binding sites ( $B_{max}$ ) were calculated from the displacement curves fitted to the equation of Akera-Cheng (2) by computer. Displacement curves were obtained by adding increasing amounts of [Nle $^{13}$ -po]motilin, EM-A, EM-523 or EM-A N-oxide to the incubation media, and the negative logarithm of the concentration displacing 50% of the label (pIC $_{50}$ ) was calculated from computer fits to these curves.

#### Contraction Studies

A segment of the distal colon 5 cm above the pelvic brim and a segment of proximal colon 3 cm distal to the ileocolonic junction were excised. The tissue was placed in oxygenated HEPES solution at 37°C. The mucosa was removed and the muscle segments were cut into strips measuring  $0.5 \times 1.5$  cm. Proximal longitudinal strips were cut from the taenia, proximal circular strips from the intertaenia region. In the distal colon this distinction could not be made as the longitudinal muscle forms a continuous outlayer.

These full thickness strips were oriented in the longitudinal or circular axis and the biological activity was tested by measuring isotonically the response to motilin ( $10^{-7}$  M), EM-A ( $2 \times 10^{-5}$  M) and EM-523 ( $10^{-6}$  M). The response was expressed relative to the maximum obtained with acetylcholine (ACh) ( $10^{-4}$  M). The effect of blocking agents was studied by incubating segments for 10 min prior to a challenge with EM-A. Cumulative dose-response curves were established by adding increasing amounts of compound and the negative logarithm of the concentration giving 50% of the maximum contractile response (pED $_{50}$ ) was obtained

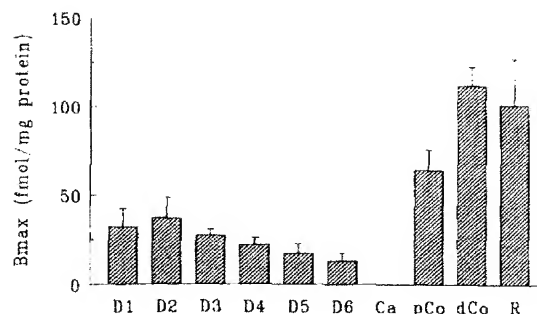


FIG. 2. Distribution of motilin receptors along the gastrointestinal tract of the rabbit. The maximum number of binding sites, expressed in fmol/mg protein, was obtained from displacement curves fitted to the equation of Akera-Cheng. Experiments were performed with crude homogenates of six consecutive 50-cm portions of the small intestine (D1-D6), caecal (Ca), proximal colonic (pCo), distal colonic (dCo) and rectal (R) tissue.

from computer fits to the curves.

#### Data Analysis

Results shown in the text or in the figures are mean values  $\pm$  S.E.M. Statistical analysis was done using analysis of variance and  $p$  values less than 5% were regarded as significant.

#### RESULTS

##### Binding Studies

Figure 2 shows the distribution of the maximum number of motilin binding sites in the different parts studied. As previously reported, motilin receptors are present in the small intestine where their density decreases aborally (4). We were unable to demonstrate binding in the caecum, but binding reappears in the proximal colon ( $B_{max}$ :  $64 \pm 12$  fmol/mg protein), where it is even more pronounced than in the duodenum. The highest receptor density is reached in the distal colon ( $B_{max}$ :  $112 \pm 11$  fmol/mg protein), where it is almost 4 times the density found in the duodenum. The affinity of motilin for its receptor is comparable in all regions studied. Analysis of variance of the  $K_d$  values for the different regions did not reveal significance,  $F(8,46) = 1.40$ ,  $p > 0.05$ , and the overall mean was  $1.10 \pm 0.22$  nM.

The displacement curves obtained by incubating distal colonic smooth muscle membranes with [ $^{125}$ I]-[Nle $^{13}$ -po]motilin and increasing concentrations of unlabeled [Nle $^{13}$ -po]motilin, EM-523, EM-A and EM-A N-oxide are shown in Fig. 3. Similar experiments were also performed with proximal colonic and duodenal tissue. From the displacement curves the concentrations required to reduce binding by 50% were derived and these data are summarized in Table 1. For this parameter, too, analysis of variance did not reveal any significant difference between the different tissues and although the potencies of the compounds were clearly different the order of potency was in every tissue the same (motilin > EM-523 > EM-A > EM-A > N-oxide).

##### Contraction Studies

An example of the contractile response of proximal colonic smooth muscle strips towards  $10^{-7}$  M [Nle $^{13}$ -po]motilin,  $10^{-6}$  M EM-523 and  $2 \times 10^{-5}$  M EM-A is given in Fig. 4. When the response reached a plateau level, a supramaximal dose of ACh

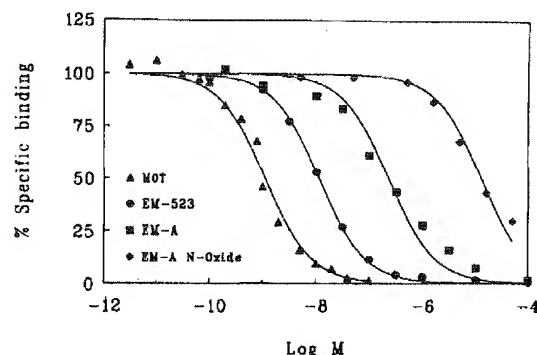


FIG. 3. Displacement of  $^{125}\text{I}$ -[Nle $^{13}$ -po]motilin bound to a crude membrane preparation from distal colonic smooth muscle tissue by unlabeled [Nle $^{13}$ -po]motilin, EM-523, EM-A and EM-A N-oxide.

( $10^{-4}$  M) was given and the response to motilin or the motilides was expressed relative to this maximum. While only an average response of 24% (motilin:  $21 \pm 3\%$ , EM-523:  $19 \pm 3\%$ , EM-A:  $31 \pm 2\%$ ) was obtained in longitudinal muscle, it amounted to 81% in strips suspended in the circular axis (motilin:  $80 \pm 3\%$ , EM-523:  $78 \pm 4\%$ , EM-A:  $84 \pm 2\%$ ). The intrinsic activities of motilin, EM-523 and EM-A did not differ from each other.

Similar results were obtained in strips cut from distal colon. An example is shown in Fig. 5. Although the response in longitudinal strips was higher (motilin:  $49 \pm 4\%$ , EM-523:  $54 \pm 6\%$ , EM-A:  $48 \pm 5\%$ ) than the 24% obtained in proximal longitudinal colon, the response in circular strips was not significantly different (motilin:  $72 \pm 4\%$ , EM-523:  $72 \pm 6\%$ , EM-A:  $75 \pm 5\%$ ).

The  $\text{pED}_{50}$  values, calculated from the dose-response curves, are summarized in Table 2. Similar to the binding studies, there was no difference in potency for each compound between duodenum, circular proximal colon and circular distal colon, and the order of potency was the same as in the binding studies.

#### Pharmacological Studies

The effects of TTX ( $10^{-6}$  M), atropine ( $10^{-6}$  M) and verapamil ( $10^{-6}$  M) on the contractions induced by EM-A and ACh in distal colonic tissue oriented in the circular direction are shown in Fig. 6. TTX, which by itself induced a contraction, was unable to block an EM-A ( $2 \times 10^{-5}$  M)-induced contraction, or the effect of a subsequent administration of ACh ( $10^{-4}$  M). Atropine had no effect upon EM-A but it inhibited, as expected, the response to ACh. Verapamil caused a small relaxation and inhibited almost completely both responses.

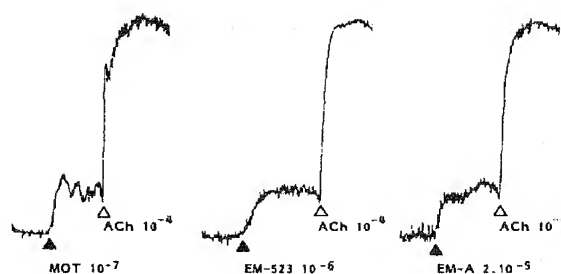
#### Additional Binding Studies

Because of the pronounced difference in intrinsic activity of

TABLE 1  
 $\text{pIC}_{50}$  VALUES OBTAINED FROM DISPLACEMENT CURVES  
IN BINDING STUDIES

	Duodenum	Proximal Colon	Distal Colon
Motilin	$8.82 \pm 0.04$	$8.89 \pm 0.04$	$8.90 \pm 0.05$
EM-523	$8.01 \pm 0.07$	$7.92 \pm 0.12$	$7.92 \pm 0.09$
EM-A	$6.71 \pm 0.09$	$6.65 \pm 0.20$	$6.68 \pm 0.04$
EM-A N-oxide	$4.69 \pm 0.20$	$4.59 \pm 0.24$	$4.82 \pm 0.19$

#### PROXIMAL COLON (long.)



#### PROXIMAL COLON (circ.)

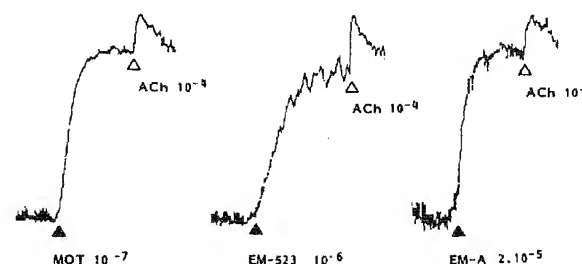


FIG. 4. Responses of proximal colonic smooth muscle strips oriented in the longitudinal (top tracings) or circular (bottom tracings) axis, towards maximally effective concentrations of motilin (MOT), EM-523 and EM-A. The agonists were administered at the point indicated by a closed triangle (▲). When the response reached its plateau an additional stimulation with acetylcholine was given (Δ).

the different compounds tested between longitudinal and circular strips in the proximal colon, tissue from the taenia and the inter-taenia region was dissected, homogenized and used in binding studies. Maximum binding was higher in the region containing predominantly circular smooth muscle:  $76 \pm 7$  fmol/mg protein versus  $42 \pm 11$  fmol/mg protein for the taeniated part.

#### DISCUSSION

Our study indicates that in the rabbit the target region for motilin is not restricted to the upper gut, but extends to the colon. Receptor density is highest in the distal colon, the most important contractile response is obtained in proximal and distal circular smooth muscle strips. The affinity of the receptor is identical in all organs studied and the persistence of the response in the presence of TTX suggests that the receptors are present on the smooth muscle cells itself.

We have previously shown that motilin receptor density decreases aborally in the rabbit small intestine (4). In the present study this distribution was studied in more detail, also for comparative purposes. We confirm the existence of a receptor density gradient in the small intestine, which correlates with the decrease in the contractile response which we recently explored for motilin and EM-523 (6). Although motilin's mechanism of action

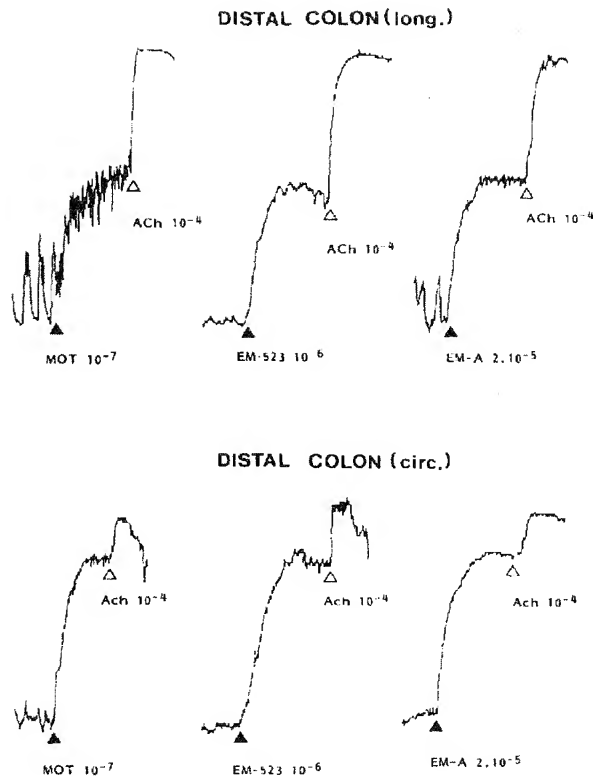


FIG. 5. Responses of distal colonic smooth muscle oriented in the longitudinal (top tracings) or circular axis (bottom tracings) towards motilin, EM-523 and EM-A. The experimental protocol was the same as for Fig. 4.

seems to differ in rabbit and in dog (7) a similar decrease in sensitivity has been described in dog. Thus in dog motilin infusions are able to induce phase 3 activity in the proximal gut but not in jejunal denervated loops (15). Close arterial infusions of motilin also demonstrate a decreased responsiveness of the canine lower small intestine (7), and when divided into four isolated segments, motilin was only able to induce MMCs in the first three and within these three the sensitivity for motilin decreased distally (10). In man, too, evidence for an aborally decreasing sensitivity has been presented (12).

In contrast to the situation in the small intestine, motilin receptor density is higher in the distal colon and the rectum than in the proximal colon. However, this is not reflected in the extent

TABLE 2  
pED<sub>50</sub> VALUES CALCULATED FROM DOSE-RESPONSE CURVES IN CONTRACTION STUDIES

	Duodenum (long.)	Proximal Colon (circ.)	Distal Colon (circ.)
Motilin	8.25 ± 0.16	8.03 ± 0.10	7.99 ± 0.04
EM-523	7.75 ± 0.16	7.55 ± 0.03	7.53 ± 0.28
EM-A	5.91 ± 0.20	5.84 ± 0.04	5.77 ± 0.05

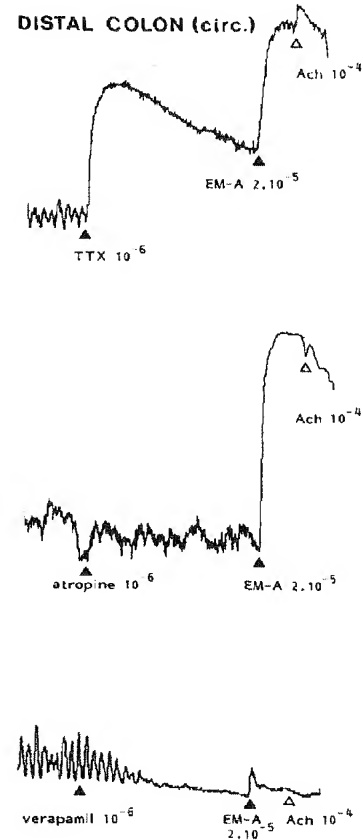


FIG. 6. Effect of  $10^{-6}$  M TTX (top),  $10^{-6}$  M atropine (center) and  $10^{-6}$  M verapamil (bottom) on the contractile response towards  $2 \times 10^{-5}$  M EM-A and a cumulative stimulation with  $10^{-4}$  M acetylcholine. After a stabilization period the blocking agents were added at the point indicated by a closed triangle (▲). Ten min later EM-A was administered (second ▲) and about 2 min 30 s later acetylcholine (Δ).

of the contractile response, although within the proximal colon the more prominent response of the circular muscle may be related to the higher receptor density found in homogenates prepared from haustra compared to those prepared from the taenia. It is clear, therefore, that receptor density and biological response are related, but not truly proportional to each other. There may be several reasons for this. Contractile strength was determined in relation to the acetylcholine response and the latter may vary along the gastrointestinal tract. This seems unlikely because only small variations in active tension generated by acetylcholine have been reported in the small intestine (19) and colon (23). Motilin receptor density was expressed relative to protein content (as is usually done in receptor studies) but the use of a variable related to the amount of the contractile apparatus may be more meaningful.

Few attempts have been made to relate receptor parameters to the extent of the biological response. It is even accepted that both may not correlate because of the existence of spare receptors. Ringer et al. (18) suggested that the rabbit colon contained a large number of spare cholinergic receptors, because of the difference in the time course of the response in binding and contraction experiments, and such a difference is also found in our experiments.

Although a large population of spare receptors for motilin may explain the large receptor density found in the colon, it is clear that the differences and correlations which we observed suggest that the response to motilin may be a good model to study the effect of receptor parameters upon biological response.

Although a motilin antagonist has not been described yet, the good correlation between the ability of erythromycin derivatives to displace bound motilin and to induce contractions is an indication that erythromycin is a motilin agonist (5,14). This is again confirmed in this paper and binding and contractility parameters are comparable to those obtained in the upper gut (5, 6, 14). Apparently there is only one motilin receptor.

Differences in the response of proximal and distal, circular and longitudinal smooth muscle may be related to anatomical differences. The rabbit colon has been considered an interesting model because the proximal colon is taeniated, the distal colon is not taeniated. In other species the whole colon may be taeniated (man) or not (cat). Strunz et al. (22) noted that motilin had an effect on the taenia coli of human colon, while in the rabbit only circular muscle responded. However, our data clearly show that both the circular and longitudinal muscle layers of the proximal taeniated and the distal nontaeniated rabbit colon respond to motilin, but the response of circular muscle is more important.

Apart from the anatomical differences between proximal and distal colon, there is a difference in function. The proximal colon allows water absorption, whereas the distal colon functions as a temporary storage area. As yet these functional differences have not been correlated with differences in regulatory mechanisms. Tucker et al. (23) reported that the proximal colon has a higher resting tension, and Sevy and Snape (20) found that the distal colon relies more on the influx of external calcium than the prox-

imal colon, but the importance of these findings in functional terms is unclear. This is also true for our findings of differences in sensitivity of circular and longitudinal muscle.

As in the small intestine the response to EM-A in distal colonic smooth muscle was blocked by verapamil, but not by TTX or atropine. The contraction initiated after TTX administration is a phenomenon also observed by others (21) in the rabbit. It may be due to a removal of tonic inhibition mediated through adrenergic neurons, because phentolamine has a similar effect. The persistence of the effects of the motilin agonist EM-A in the presence of these agents indicates that motilin acts directly on smooth muscle cells. That the response depends on the influx of extracellular calcium has also been found for motilin's effect on the duodenum (11). Motilin's mechanism of action appears, therefore, to be the same in the colon as in the small intestine. This is altogether a somewhat surprising finding, because the control of colonic motility is usually discussed in relation to its innervation.

The functional importance of motilin in the rabbit is completely unknown. Apparently in the small intestine and in the colon, motilin may affect motility via an endocrine mechanism. If motilin receptors are also present in the human colon, the pharmacological applications of macrolides as prokinetic agents may extend beyond a gastrokinetic effect (8) to the treatment of colonic motor disorders.

#### ACKNOWLEDGEMENTS

This work has been supported by grant 3.0048.87 from the Belgian Foundation for Medical Research (FGWO, Brussels, Belgium) and grant 86/91-99 from the Belgian Ministry of Science. The authors wish to thank L. Gregoire, L. Nijs, M. Parein, T. Thijs and S. Spee for skilful technical assistance and L. Versées for typing the manuscript.

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